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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Epstein Barr virus (EBV) infection in human is associated with variety of malignant diseases including Burkitt's Lymphoma (BL), nasopharyngeal carcinoma, Hodgkin's disease and lympho proliferative disorders of immuno suppressed patients as well as significant portion of estrogen receptor negative invasive breast cancers and large numbers of rapidly growing fibroadenomas of the breast in immuno compromised patients. In all latently infected cells EBV expresses two small non-polyadenylated RNAs (EBERs).Recent studies have shown that EBERs alone provide tumorigenic potential. We have identified that EBERs (which possess extensive secondary structure) has strong nucleotide sequence homology to the codingexon of kinesin super family of motor proteins. Kinesin is an essential member of the multiprotein ß-catenin degradation complex which includes tumor suppressor adenomatos poliposis coli (APC) and GSK3ß.ß-catenin, an activator of Wnt signaling pathway is activated in many breast cancers. We hypothesize that EBERs down regulate KIF3 protein by RNA interference mechanism and interfere with the functionality and anti-tumor activity of APC. In this proof of principle study we will analyze if EBERs indeed alter expression of KIF3 protein via RNA interference and whether such alteration provide growth advantages in mammary epithelial cells.

15. Subject Terms (keywords previously assigned to proposal abstract or terms which apply to this award)

EBV, non-coding RNA, kinesin, ß-catenin, RNA-interference, epithelial cell growth

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INTRODUCTION

Epstein Barr Virus (EBV) has been detected in significant portion of estrogen negative invasive breast cancers. EBV has also been detected in large numbers of rapidly growing fibroadenomas of the breast in immunocompromised patients.[1] Because of close association of EBV with various epithelial and lymphoid cancers it has been suggested that EBV plays important role in the genesis of this subset of breast cancers. The molecular mechanism of how EBV facilitates oncogenesis however, has remained poorly understood. In recent years several studies, including our own, reported that RNA molecules act as a coactivator or corepressor of gene transcription. Also, small interfering RNAs and micro RNAs are increasingly being identified as important regulator of gene expression. Through BLAST search we have identified that EBERs (which possess extensive secondary structure) has strong sequence homology to kinesin superfamily of motor proteins. Recently, it has been reported that association of kinesin protein KIF3 is required for the transport of tumor suppressor protein adenomatous polyposis coli (APC) along cytoplasmic microtubules.[3] Recent studies have shown that APC, whose mutation was originally identified in colorectal cancers, is also often mutated in breast cancer patients (as high as 18%).[4] APC is known to facilitate proteasomal degradation of ß-catenin, an activator of Wnt signaling pathway that is activated in many breast cancers. Suppression of kinesin has been recently shown to disrupt APC transport and facilitate nuclear accumulation of ß-catenin.[5] *We hypothesize that EBERs down regulate KIF3 protein by RNA interference mechanism and interfere with the functionality and antitumor activity of APC.* The specific aims of this proof of principle study is to analyze if EBERs indeed alter expression of KIF3 protein via RNA interference and to analyze if such alteration provide growth advantages in mammary epithelial cells. Analysis of EBER's role as a tumor suppressor antagonist is highly significant as tumorigenesis is a multistep process, which involves many factors including activation of oncogenes and inactivation of tumor suppressor genes or their products. If our hypothesis becomes true, inhibition of EBERs' expression or their inactivation could be an import therapeutic avenue to treat this subset of breast cancers and the concept may even be used for other EBV associated cancers.

BODY
To fulfill the specific aims of this study we set out six individual tasks as follows:

Task 1. Development of EBER expression system.

Task 2. Analysis of kinesin mRNA and protein expression in cells that are expressing EBV EBER.

Task 3. Analysis of activity of tumor suppressor APC in cells expressing EBER RNA.

Task 4. Identification of small interfering RNA molecules complementary to various kinesins.

Task 5. Analysis of growth properties of cells that are expressing EBER.

Task 6. Data integration and evaluation of the concept hypothesis and pilot experiments to carry the concept ahead.

In this section data obtained so far for individual tasks are presented.

Task 1
To develop a system for EBER expression in vitro we cloned the EBV genome that contain both the EBER1 and EBER2 coding frames from genomic DNA of EBV positive lymphoid cell line P3HR1. We also PCR amplified individual genes EBER1 and EBER 2.

Primers used for this purpose were (bold bases were enzyme sites):
C: CGGGGTCTC**GGAtCc**CCTAGGTCA
D: TGCCGTTTAATGAT**AGATct**CCAGGAG
1B: CCCGTTTAGGT**aGAtCT**GCGGGATAA
2A: GGGCTTAACGTT**GgATCC**CAGAAGATG
These primers were used according to the figure below (Figure 1) to amplify both EBERs (EBER12 using CD) or individual EBER1 (C-1B) or EBER2 (2A-D) in PCR amplification reaction using high fidelity Taq polymerase.
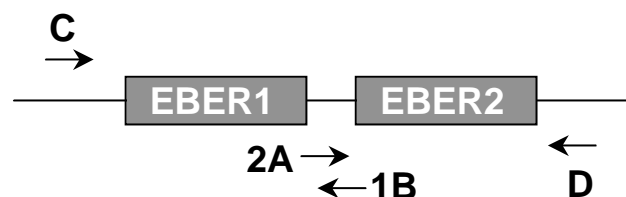

Figure 1. Schematic diagram of the PCR amplification strategy for the EBER1 and EBER2 genes.

Amplification products were purified, digested with BamHI and BglII (as appropriate), gel purified and cloned into the BamHI site of pDNA3.1 (Figure 2). The inserts of representative clones were completely sequenced to confirm that there was no PCR introduce error.
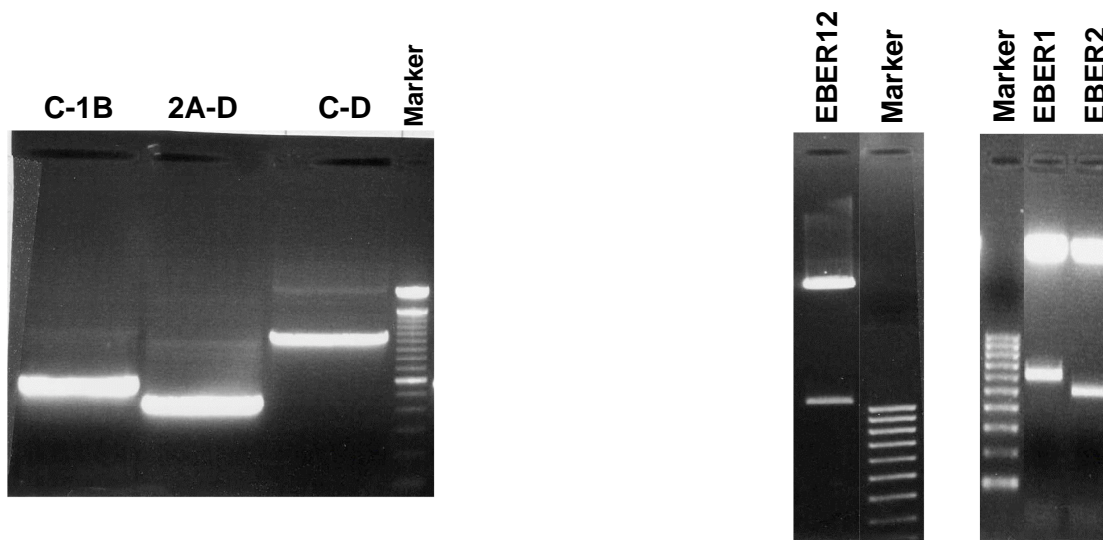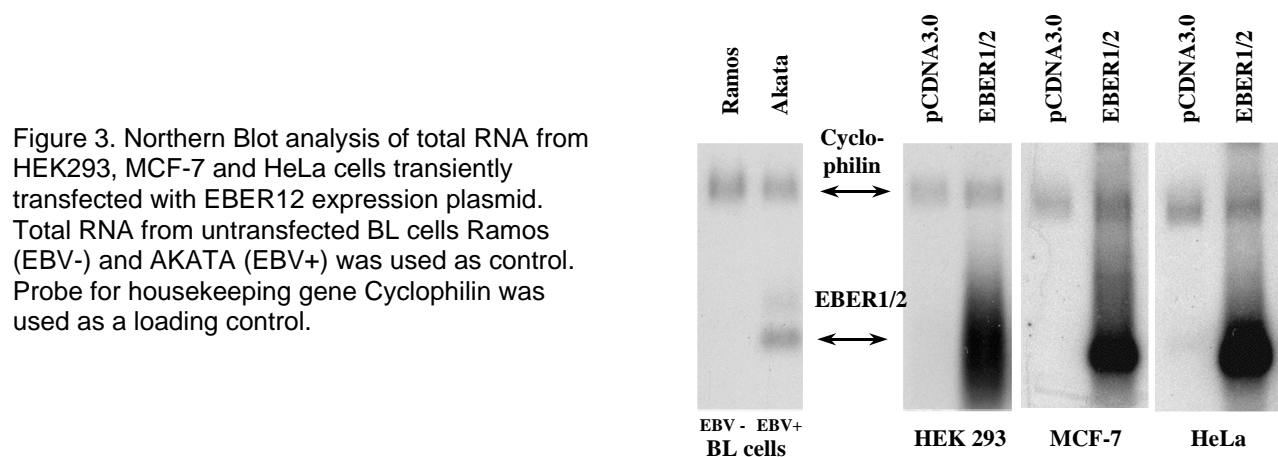

Figure 2. A. Analysis of the PCR amplification product from EBER genes. 100 bp ladder from Invitrogen was used as molecular weight marker. B. Restriction digestion analysis of the pCDNA3.1 clones. 100 bp ladder from Bio-Rad was the marker in this experiment.

To test whether these clones could express EBER RNA, they were transfected in various epithelial cell lines. Total RNA was extracted from transfected cells by Trizol extraction and analyzed by northern blot analysis. EBER1 specific sequence was PCR amplified from pCDNA-EBER1 clone and used as template for the 32P-labeled probe for northern blot analysis. Total RNA preparations from EBV positive and negative Burkitt's lymphoma cell lines (Akata and Ramos, respectively) were used as positive and negative controls, respectively. As shown in the Figure 3 below, epithelial cells HEK 293, HeLa and MCF-7, all expressed EBER abundantly demonstrating that epithelial cells support EBER production.

Production of EBER in mammary epithelial cell line MCF-10A has not been tested although that in MCF-10F has been successfully performed with the EBER12 plasmid construct. It may be noted that MCF-10A and MCF-10F both comes from same original cell stock (adherent or floating cells) and in vitro they both behave similarly. Therefore, use of MCF-10F in place of MCF-10A is not a deviation from original approved SOW.

Figure 3. Northern Blot analysis of total RNA from HEK293, MCF-7 and HeLa cells transiently transfected with EBER12 expression plasmid. Total RNA from untransfected BL cells Ramos (EBV-) and AKATA (EBV+) was used as control. Probe for housekeeping gene Cyclophilin was used as a loading control.

Task 2

To determine if Kinesin Kif3 expression level is altered in cells that are expressing EBV EBER, we analyzed Kinesis mRNA level. To get a better assessment of the effect of EBER, we decided to generate cell lines that would stably express EBER and then to test Kif3 mRNA level.

We transfected MCF-7 and HeLa cells with pCDNA-EBER12 clones and maintained them in presence of G418 drug. Several drug resistant colonies were selected, cloned and EBER expression was analyzed by northern blot analysis. As shown in Figure 4, we isolated several G418 resistant colonies from all three different cell lines that expressed high level of EBERs.
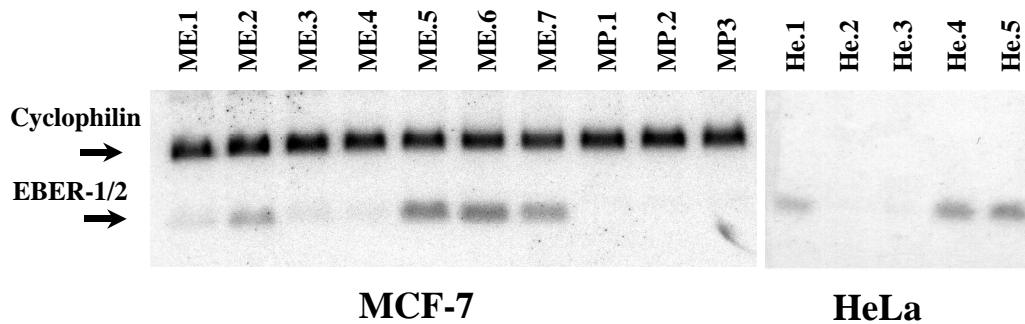
Figure 4. Generation of cell lines that stably express EBER RNA. Total RNA from these cells was analyzed for EBER RNA by northern blot analysis. Clones with a prefix 'MP' (in MCF-7) comes from vector transfection only. Probe for house-keeping gene cyclophilin was not included in northern blot of HeLa cell lines.

To test the possibility that EBER expression could affect Kinesin mRNA level, we tested one of the HeLa cell lines we established that stably expresses EBER. We isolated total cellular RNA from one such line HE.4 (see Figure 4 above) and from another HeLa line that was stably transfected with empty vector pCDNA3.1 (HP.2) and tested the Kinesin Kif3 mRNA level by northern blot analysis. A 300 bp KIF3c DNA fragment was amplified by RT-PCR from total RNA isolated from HeLa cells and used as
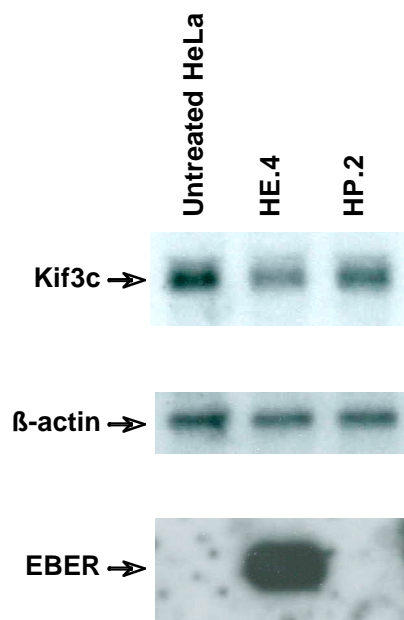

Figure 5. Expression of Kinesin Kif3c mRNA in HeLa cells stably transfected with EBER (HE.4) or with empty vector pCDNA3.1 (HP.2). Twenty microgram of total RNA from each cell was separated in formaldehyde-agarose gel analyzed by northern blotting using specific probes for Kif3c, EBER or ß-actin.

probe for northern blot analysis. Primers used for this purpose was designed from human KIF3c mRNA sequence (genbank accession number NM_002254) forward primer: 5'-GCCCAAGACCTTCACCTTTGA-3', reverse primer: 5'-TTGGAGAGCAGGTCTCGAATC-3'. As shown in Figure 5, Kif3c mRNA level was significantly lower in HE.4 line compared to HP.2 or parental HeLa line.

This data demonstrates that EBER expression in HeLa cells lower the Kinesin mRNA level although it remains to be determined yet if this effect is due to RNA interference mechanism.

KEY RESEARCH ACCOMPLISHMENTS

The first two tasks of the project have been achieved so far where we have successfully developed EBER expression system and demonstrated that EBER expression reduces Kinsin Kif3 level.

REPORTABLE OUTCOMES

Data obtained from this work was presented as a Poster in the "Era of Hope" Breast Cancer Meeting organized by Department of Defense, which was held in Pennsylvania Convention Center, Philadelphia, June 8-11, 2005.

Title: ROLE OF EPSTEIN-BARR VIRUS ENCODED SMALL RNAS IN BETA-CATENIN DEGRADATION
Authors: Sajal K. Ghosh and Idowu Akinsheye
Abstract:
Significant improvement of breast cancer survival rate in recent years has been possible because of our better knowledge of the risk factors for the disease and molecular mechanism of the abonormalities as well as, early detection. In recent years, Epstein Barr Virus (EBV) has been detected in significant portion of estrogen receptor negative invasive breast cancers. EBV has also been detected in large numbers of rapidly growing fibroadenomas of the breast in immuno-compromised patients. EBV is associated with variety of malignant diseases including Burkitt's Lymphoma (BL), nasopharyngeal carcinoma, Hodgkin's disease and lymphoproliferative disorders of immunosuppressed patients. Because of this close association it has been suggested that EBV plays important role in the genesis of EBV associated breast cancers. Although most of the EBV genes are not expressed in EBV associated tumors, two non-coding RNA molecules (EBERs) are expressed consistently. Recent studies have suggested that these EBERs may have oncogenic potential. We have identified that EBERs (which possess extensive secondary structure) has strong sequence homology to kinesin superfamily of motor proteins. Suppression of kinesin protein (KIF3C) has been recently shown to disrupt adenomatous polyposis coli (APC) transport and facilitate nuclear accumulation of beta-catenin. ß-Catenin is an important activator of Wnt signaling pathway that is activated in many breast cancers. We hypothesize that EBERs down regulate KIF3 protein by RNA interference mechanism and interfere with the functionality and antitumor activity of APC.
In order to test our hypothesis, we have cloned the EBER genes in pCDNA 3.1 vector. In transient transfection experiments in epithelial cells such as HeLa, human embryonic kidney cells 293 and also in human breast cancer cell line MCF-7, followed by northern blot analysis, we confirmed that these constructs make large amount of EBERs. Next, we analyzed KIF3 gene expression in EBER expressing cells by northern blotting. A 350 base pair cDNA segment of KIF3 mRNA was amplified from total cellular RNA from HeLa cells and was used as probe for this purpose. Our preliminary data indicate that KIF3C mRNA level was significantly lower in HeLa cells that were expressing EBER but not in cells that were transfected with empty pCDNA vector. We are currently testing how this apparent reduction in KIF3C level affects ß-catenin degradation in a transient ß-catenin dependent reporter assay system. Using commercially available antibodies, we are also analyzing KIF3 protein expression in cells that are expressing EBER. Analysis of EBER's role as a tumor suppressor antagonist is highly significant as tumorigenesis is a multistep process which involve many factors including activation of oncogenes and

inactivation of tumor suppressor genes or their products. Detail results of these studies will be presented in the meeting.

CONCLUSIONS

The information obtained so far is already a significant support for the original concept that we put forward that EBER may interfere with Kinesin expression by a RNA interference mechanism. The inability of the Kinesin to work properly could ultimately inhibit function of tumor suppressor APC and thus to control unwanted accumulation of pro-tumorigenic protein ß-catenin.

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APPENDICES

None

SUPPORTING DATA

None